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Genomic organization and chromosomal localization of the murine 2 P domain potassium channel gene *Kcnk8*: conservation of gene structure in 2 P domain potassium channels ☆

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Abstract

A 2 P domain potassium channel expressed in eye, lung, and stomach, Kcnk8, has recently been identified. To initiate further biochemical and genetic studies of this channel, we assembled the murine *Kcnk8* cDNA sequence, characterized the genomic structure of the *Kcnk8* gene, determined its chromosomal localization, and analyzed its activity in a *Xenopus laevis* oocyte expression system. The composite cDNA has an open reading frame of 1029 bp and encodes a protein of 343 amino acids with a predicted molecular mass of 36 kDa. Structure analyses predict 2 P domains and four potential transmembrane helices with a potential single EF-hand motif and four potential SH3-binding motifs in the COOH-terminus. Cloning of the *Kcnk8* chromosomal gene revealed that it is composed of three exons distributed over 4 kb of genomic DNA. Genome database searching revealed that one of the intron/exon boundaries identified in *Kcnk8* is present in other mammalian 2 P domain potassium channels genes and many *C. elegans* 2P domain potassium channel genes, revealing evolutionary conservation of gene structure. Using fluorescence in situ hybridization, the murine *Kcnk8* gene was mapped to chromosome 19, 2B, the locus of the murine *dancer* phenotype, and syntenic to 11q11-11q13, the location of the human homologue. No significant currents were generated in a *Xenopus laevis* oocyte expression system using the composite *Kcnk8* cDNA sequence, suggesting, like many potassium channels, additional channel subunits, modulator substances, or cellular chaperones are required for channel function.

Keywords: Fluorescence in situ hybridization; Intron/exon boundary

1. Introduction

Potassium channels are a diverse group of proteins with wide cellular and tissue distribution that share the common feature of high selectivity for potassium as the permeating ion. They serve a broad range of functions, influencing resting membrane potential, frequency and duration of action potentials, and various signal transduction events, thereby participating in a wide variety of cellular processes (Hille, 1997; Jan and Jan, 1997). Defects in potassium channels have been associated with a diverse array of human genetic disease including neuromuscular disorders, cardiac arrhythmias, seizures, neonatal hypoglycemia, and hypertension (Ackerman and Clapham, 1997; Sanguinetti and Spector, 1997). Given the broad spectrum of functional capacities subserved by potassium channels, it is not surprising that they exhibit marked genetic, biochemical, and structural diversity (Coetzee et al., 1999).

Potassium channels share a common pore-forming P domain that lines the ion-conducting pathway and is critical for determining channel conductance, selectivity, and sensitivity to open-channel blockade. A novel family of potassium channels has been identified that contains 2 P domains and 4 transmembrane domains in tandem (2P/4TM) (Ketchum et al., 1995; Goldstein et al., 1996; reviewed in Goldstein et al., 1998). Members of this family, like potassium channels in general, differ in their cellular and tissue

Abbreviations: Fish, fluorescence in situ hybridization; P, pore domain; RACE, rapid amplification of cDNA ends; TM, transmembrane domain

☆ The sequences reported in this paper have been deposited in the GenBank database (Accession number AF158234).

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Table 1
Oligonucleotide primers

A	5′-CCCAAGGGCTGCATAGACCA-3′
B	5′-CAGCAGGTGGGCCATAAGCAGGAGCAG-3′
C	5′-CCTGCACCCAGCCATTTACC-3′
D	5′-ACCGATGAAGATCAAGATGGCATC-3′
E	5′- CTGAAACCATGGGCCCGATACCTGC-3′
F	5′- GCCTGGAAGCTAGCCAGTTCAG-3′

distribution as well as in their biochemical, structural, and functional characteristics.

Recently, a 2 P domain potassium channel expressed in eye, lung, and stomach, Kcnk8, has been identified (Salinas et al., 1999). To initiate further biochemical and genetic studies of murine Kcnk8, we assembled the cDNA sequence, which contained significant variations from the published sequence. We cloned the chromosomal gene of murine *Kcnk8,*characterized its genomic structure, and determined its chromosomal localization by fluorescence in situ hybridization. Using our cDNA sequence, we analyzed Kcnk8 activity in a *Xenopus laevis* oocyte expression system. One of the intron/exon boundaries identified in the region encoding the first P domain of *Kcnk8* is present in all other mammalian members of the 2 P domain potassium channel gene family available in current databases and in 20 of 36 *C. elegans* 2 P domain potassium channel genes (Wang et al., 1999), revealing significant evolutionary conservation of gene structure.

2. Materials and methods

2.1. Rapid amplification of cDNA ends

5′ and 3′ rapid amplification of cDNA ends (RACE) were performed using primers A and B for 5′ RACE and C and D for 3′ RACE (Table 1) as described (Edwards et al., 1991) with murine brain RNA as template. Amplification products were subcloned and sequenced.

2.2. Genomic clone detection and analyses

A murine genomic library in PACs was screened with two oligonucleotide primers, E and F (Table 1), as described (Pierce et al., 1992). These primers correspond to the beginning of the coding sequence of *Kcnk8* cDNA and amplify a 146 bp product from murine genomic DNA.

2.3. Fluorescence in situ hybridization

Metaphase chromosomes were prepared by standard techniques. The *Kcnk8* genomic clone was labeled with digoxigenin-11-dUTP by nick translation. A murine chromosome 19 probe ATCC# 63397 (Mongelard et al., 1996) was similarly labeled with biotin. Hybridization conditions, post hybridization washes, and probe detection were performed as previously described. Probes were detected with rhodamine-antidigoxigenin and FITC-avidin antibodies as described (Boyle et al., 1992; Ried et al., 1992).

Images were captured using a computer controlled Zeiss Axioskop epifluorescence microscope coupled to a CCD camera. FTIC, rhodamine and DAPI fluorescent signals were recorded separately as gray scale images, enhanced, pseudo-colored and merged.

2.4. cRNA synthesis

The expression vector pRAT was generated by inserting a multiple cloning site, flanked by the 5′ and 3′ untranslated regions of *Xenopus laevis* β-globin in the *Nhe*I and *Pme*I sites of pCRIII (Invitrogen, Carlsbad, CA). *Kcnk8*cDNA was inserted into the *EcoR*I and *Bgl*II sites of the multiple cloning site of pRAT. This plasmid construct was linearized with *Not*I for in vitro transcription with T7 RNA polymerase (Ambion, Austin, TX). Transcript concentration was quantified by spectrophotometer and compared with control samples separated by agarose gel electrophoresis. Aliquots were stored at −80°C.

2.5. Electrophysiology

Oocytes were isolated from *Xenopus laevis* frogs (Nasco, Atkinson, WI), defolliculated by collagenase treatment, and injected the same day with 46 nl of cRNA solution containing 1–20 ng of cRNA encoding either *Kcnk8*, various other potassium channels (see below), or a mixture of both.

Whole cell currents were measured 1–5 days after cRNA injection by two-electrode voltage clamp using an oocyte clamp (Warner, Hamden, CT). Data were sampled at 5 kHz and filtered at 1 kHz. Electrodes, made from borosilicate glass tubes (Garner glass, Claremont, CA), contained 3 M KCl and had resistances of 0.2–1 MΩ. Recordings were performed under constant perfusion at room temperature. Bath solutions were 5 mM KCl solution (in 95 mM NaCl, 5 mM KCl, 1 mM MgCl, 0.3 mM $CaCl_2$, 5 mM HEPES – pH 7.5) or 20, 50 and 100 mM KCl solutions in which KCl is isotonically substituted for NaCl.

2.6. Computer analyses

Computer-assisted analyses of derived nucleotide and predicted amino acid sequences were performed utilizing the sequence analysis software package Lasergene™ (Madison, WI) and the BLAST algorithm, National Center for Biotechnology Information (Bethesda, MD).

3. Results

3.1. Murine Kcnk8 cDNA sequence

A homology search of the BLASTN database was performed using the coding sequence of the human 2 P domain potassium channel *KCNK1* (hOHO-1) as query

(GenBank Accession number U76996) (Goldstein et al., 1998). This search identified one expressed sequence tag, W18545 that included a single P loop motif. 5′ and 3′ RACE were performed with murine brain RNA as template. Together, the sequences obtained from RACE and the EST identified an open reading frame of 1029 bp. The sequences around the predicted translation start site, GCTATGG, match important consensus sequences, specifically, there is a G in position −3 and a G in position +4 (Kozak, 1986). A termination codon was identified 39 bp upstream of the initiator methionine in the 5′ untranslated region.

Deduced amino acid sequence of the open reading frame predicts a peptide of 343 amino acids with a predicted molecular mass of 36 kDa and a pI of 5.8. Secondary structure analyses predict 2 P domains bounded by hydrophobic segments and four potential transmembrane helices. Sequences encoding a potential single EF-hand motif and four potential SH3-binding motifs are present in the intracellular COOH-terminus of the protein.

Compared to the *Kcnk8* cDNA sequence recently reported by Salinas et al. (1999), we identified several sequence differences which were verified by comparison of our cDNA sequence with the sequence obtained from a *Kcnk8* genomic clone (see below). At cDNA position 248, (numbering from +1 A of the ATG initiator methionine), we found A for G, which changes a serine to glycine. Most importantly, at cDNA position 1003, we found a frameshift, GGA CCT GAG.. instead of GGA CCT *T*GA Stop, thus our predicted protein is extended by 8 amino acids. Our genomic clone sequence verified our cDNA findings. Four nucleotide differences were found in the 3′untranslated region: position 1050 – G for A, 1065 – T for G, 1090 – A for C, and 1100 delete T. In addition, our cDNA extends beyond position 1113, where the cDNA sequence reported previously ends. There is a polyadenylation signal 1153 bp downstream of the termination codon. The RNA and genomic clones used in these experiments were obtained from 129 Sjv mice; the strain of mice used by Salinas and colleagues (1999) is not reported. Based on the above data, it seems unlikely that these sequence variations are due to strain differences, but this possibility cannot be completely excluded.

3.2. Genomic cloning and structure of Kcnk8

The PCR-based screening of the murine genomic DNA PAC library yielded one PCR-positive clone ~100 kb in length. DNA fragments that hybridized to the screening probe were purified and subcloned into plasmid vectors. Restriction enzyme analysis, Southern blotting, and limited nucleotide sequencing of these subclones identified them as *Kcnk8* gene-specific. The region of this clone containing the *Kcnk8* gene, including 5′ and 3′ untranslated sequences, the introns and exons, and the 5′ and 3′flanking DNA, was sequenced on both strands.

Comparison of cDNA and genomic DNA sequences

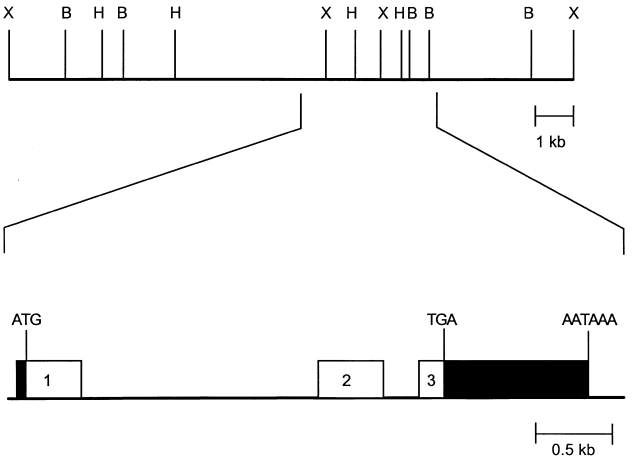

Fig. 1. Genomic organization of the murine *Kcnk8* gene. Top. A partial restriction map for the murine *Kcnk8* gene is shown: X, *Xba*I; B, *Bam*HI; and H, *Hind*III. Bottom. The coding region of the *Kcnk8* gene is contained in ~4 kb of DNA and is composed of three exons, shown in the boxes. Shaded regions represent untranslated sequences.

allowed determination of the genomic structure of the *Kcnk8* gene, a three exon gene spread over ~4 kb of DNA (Fig. 1, Table 2). The first and third exons contain untranslated sequence. Comparison of the exon/intron boundaries with reported consensus sequences reveals that the ag:gt rule was not violated at any splice junction (Horowitz and Krainer, 1994). There are no AG dinucleotides within the 15 bp upstream of the 3′ (acceptor) splice junctions.

An alternative splice variant that included the second intron in the *Kcnk8* cDNA was identified by 3′ RACE. This variant, identified in 20% of 3′ RACE subclones, leads to a frameshift and premature chain termination. Deduced amino acid sequence of this variant predicts a truncated protein that includes both P domains, but lacks part of the fourth transmembrane domain and the putative calmodulin and SH3 domain binding sites. This splice variant may represent an alternately spliced transcript or an incompletely processed mRNA.

3.3. Chromosomal localization

To localize the murine *Kcnk8* gene by fluorescence *in situ*

Table 2
Intron/exon boundaries of the Murine *Kcnk8* Gene

3′Acceptor site (c/t)11n(c/t)ag:G	Exon Eukaryotic consensus[a]	5′Donor site (C/A)AG:gt(a/g)a
5′ untranslated	1	ACCACCACCGgtaggcacta ThrThrThrG
tcttttccagGTTATGGCCA lyTyrGlyHi	2	GCACTTCTTGgtgagtccag AlaLeuLeuG
ttctccccagGTTACTTGCT lyTyrLeuLe	3	3′untranslated

[a] From Horowitz and Krainer (1994).

hybridization, the *Kcnk8* genomic clone was labeled with digoxigenin and hybridized to metaphase chromosomes. Twenty-six metaphase spreads were randomly merged and examined. The probe gave a specific hybridization signal only on chromosome 19. The hybridization locus was identified by merging fluorescent images of the probe signals with DAPI staining (G banding). The gene for *Kcnk8* localized close to the centromere at 19B (Fig. 2). Localization to chromosome 19 was confirmed by co-hybridization with a biotin-labeled murine chromosome 19 probe (Fig. 2). The murine disorder *dancer*, which is characterized by circling and head-tossing behavior, defects in hearing, and abnormal coat pigmentation, maps to this region (Blake et al., 2000).

3.4. Electrophysiology

Our cDNA sequence differed from previously published sequence, leading to a shift in the reading frame and a change in the COOH-terminal sequence. Because variations in the COOH-terminus of many potassium channels leads to critical changes in function (or lack thereof), we wanted to examine if our *Kcnk8* cDNA sequence led to differences in channel function. Thus to examine channel activity directed by this putative potassium channel gene, cRNA transcribed from *Kcnk8* was injected into *Xenopus laevis* and currents examined via a two-electrode voltage clamp technique. Assays were performed at times 1–4 days post injection. No detectable currents were identified when amounts of *Kcnk8* cRNA varying from 1–15 ng were injected into *Xenopus* oocytes (Fig. 3).

Many potassium channels require additional subunits or regulatory substances for function. Co-injection of *Kcnk8* cRNA into *Xenopus laevis* oocytes with cRNA from the following potassium channel subunits, *Kcnk1* (hOHO-1/TWIK-1) (Goldstein et al., 1998; Lesage et al., 1996), *Kcnk3* (OAT-1/TASK) (Lopes et al., 2000; Duprat et al., 1997), *KCNK2* (hTPKC1/TREK) (Goldstein et al., 1998), and *ROMK1* (Ho et al., 1993), failed to produce new currents.

The presence of a potential protein kinase C (PKC) site in the COOH-terminus suggested a regulatory role for phosphorylation in Kcnk8 regulation. The addition of either phorbol 12-myristate 13-acetate (PMA) or staurosporine, a PKC inhibitor, did not lead to detectable currents in the *Xenopus* expression system. Similar results were obtained after the addition of arachidonic acid, a regulator of the 2P/4TM potassium channel KCNK4 (Maingret et al., 1999).

The presence of a putative EF-hand domain in the COOH-terminus of Kcnk8 suggested possible modulator influence by calcium. After *Kcnk8* cRNA injection, neither treatment of the oocytes with the calcium ionophore A23187 (10–50 μM), nor direct injection of calcium into

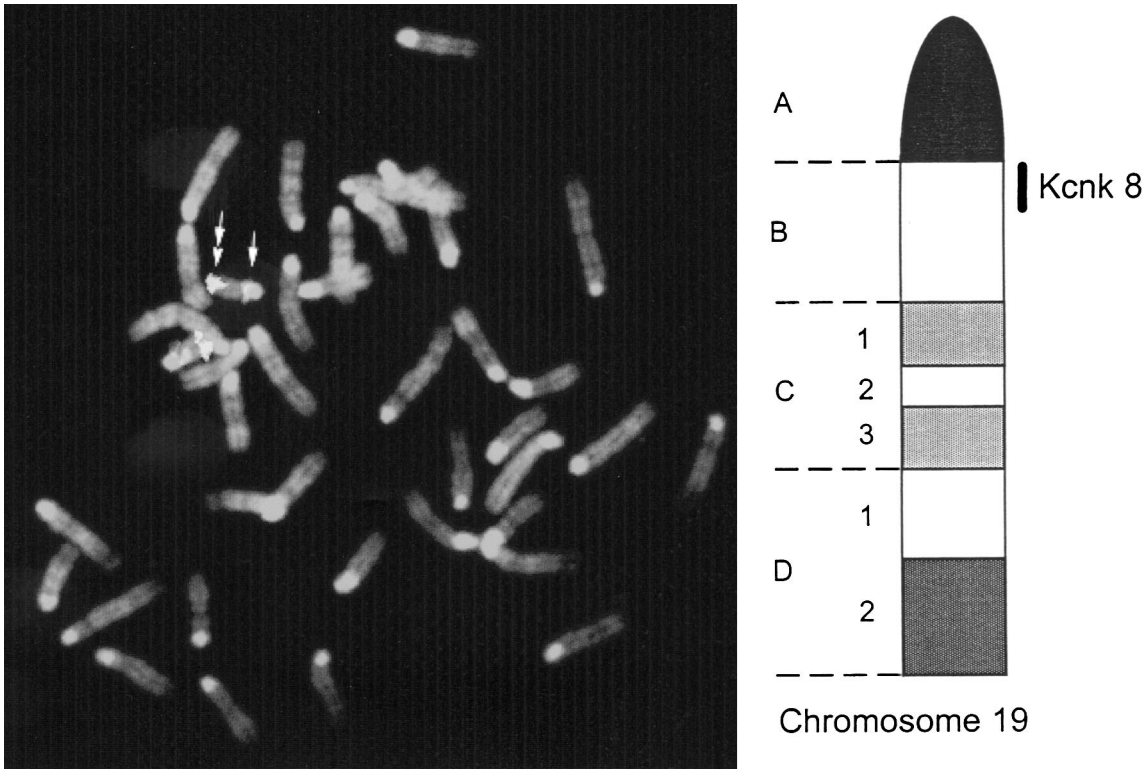

Fig. 2. Chromosome mapping of the murine *Kcnk8* gene. In situ hybridization of a digoxigenin-labeled *Kcnk8* genomic probe to DAPI-stained murine metaphase cells from mitogen-stimulated splenocytes. A. Specific labeling was observed close to the centromere at 19B (arrow). Confirmation of localization to chromosome 19 was obtained by co-hybridization with a biotin-labeled murine chromosome 19 probe (double arrow). B. Ideogram of mouse chromosome 19 indicating the localization of the murine *Kcnk8* gene to the border of band 2B.

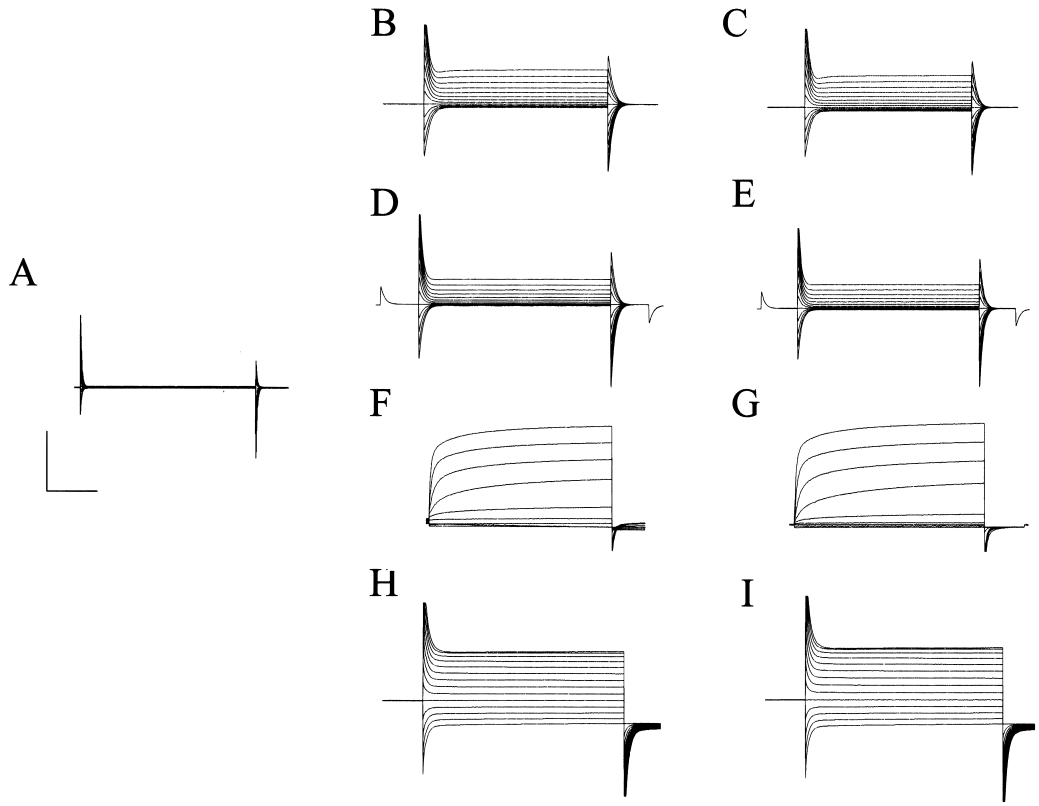

Fig. 3. Lack of expression of Kcnk8 currents in *Xenopus* oocytes. Current traces induced by: (A) Kcnk8; (B) KCNK2; (C) KCNK2 coinjected with Kcnk8; (D) KCNK4; (E) KCNK4 coinjected with Kcnk8: (F) KVLQT1; (G) KVLQT1 coinjected with Kcnk8; (H) ROMK1; and (I) ROMK1 coinjected with Kcnk8. Oocytes were perfused with a 5 mM KCl solution, containing (in mM): 95 NaCl, 5 KCl, 1 MgCl, 0.3 CaCl, 5 HEPES, pH 7.5 (see text for details). Scale bars are 5 μA and 200 ms.

the cell (raising intracellular calcium concentration by 1–50 μM), produced currents different from uninjected control oocytes. Together, these data agree with those of Salinas and colleagues (1999) who were unable to generate channel activity with Kcnk8 alone in either *Xenopus* oocytes or transfected COS cells.

3.5. Genetic analyses

The maximum likelihood of the relationship between the predicted mammalian 2P/4TM domain potassium channel genes available in the databases was determined using the amino acid sequence of the entire predicted open reading

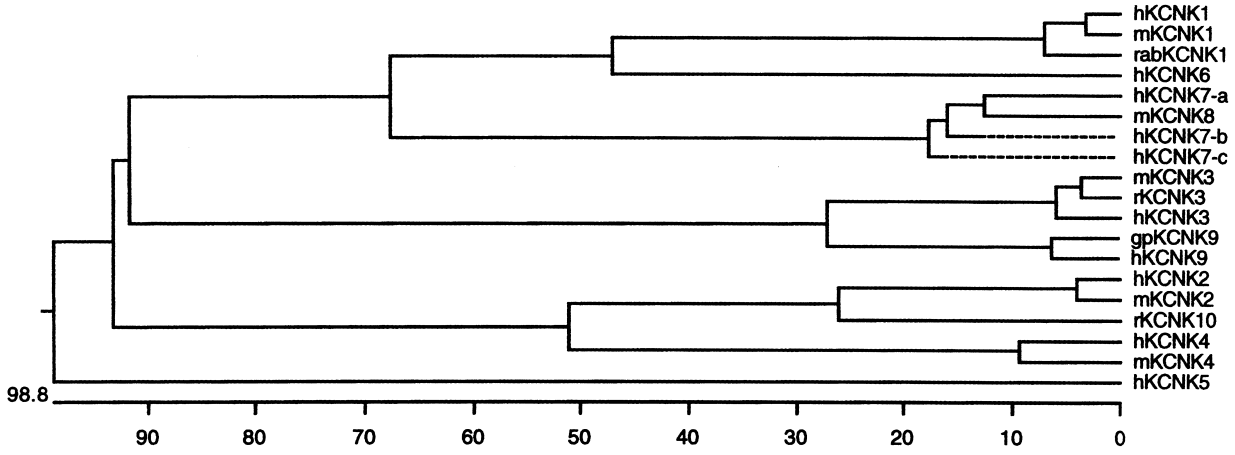

Fig. 4. *Phylogenetic tree of mammalian 2P/4TM potassium channel genes*. Following a multiple Clustal W 1.6 alignment using the predicted amino acids sequences, the standard CLUSTREE algorithm was used for computation. Abbreviations: h, human; m, murine; rab, rabbit; r, rat; gp, guinea pig. Isoforms of human KCNK7 generated by alternative splicing are denoted a, b, and c.

Table 3
Chromosomal localization and conserved intron/exon boundary in the first P domain of mammalian 2P/4TM potassium channels

Channel	Protein	Species[a]	Location	Boundary[b]	
KCNK1	hOHO, TWIK1, DPK	H	1q42–q43	SerThrThrG	lyTyrGlyHi
				TCCACCACAGgtagggtatc	ctccttgcagGTTATGGCCA
		M	8	–	–
KCNK2	TPKC1, TREK1	H	1q41	–	–
		M	19B	–	–
KCNK3	OAT1, TASK1	H	2p23.3–p24.1	ThrThrIleG	lyTyrGlyHi
				ACCACCATCGgtaacggctc	tttcccccagGCTATGGTCA
		M	5B	ThrThrIleG	lyTyrGlyHi
				ACCACCATCG	GCTATGGTCA
KCNK4	TRAAK	H	11q13	ThrThrIleG	lyTyrGlyAs
				ACCACCATCGgtgggggaga	ctctgcccagGCTATGGCAA
		M	proximal 5	–	–
KCNK5	TASK2	H	6p21	–	–
KCNK6	TOSS, TWIK2	H	19q13.1	ThrThrValG	lyTyrGlyTh
				ACCACCGTGGgtacgtaagc	actcccctagGCTATGGGTA
KCNK7		H	11q13	ThrThrIleG	lyThrGlyHi
				ACCACCACAGgtaagggagc	tcttctccagGTTATGGCCA
KCNK8		M	19B	ThrThrThrG	lyTyrGlyHi
				ACCACCACCGgtagacacat	tcttttccagGTTATGCCCA
KCNK9	TASK3	H	8	ThrThrIleG	lyTyrGlyHi
				ACCACCATAGgtaagggctgg	cttcccacagGTTACGGGCA
KCNK10	TREK2	H	14[c]	ThrThrIleG	lyThrGlyAs
				ACGACCATAG	agGGTATGGGAA

[a] Abbreviations: H, human; M, mouse.
[b] Boundaries without intron sequence are from published reports. Corresponding genomic sequences were not available at the time of submission.
[c] Assignment from Bang et al. (2000).

frames for analysis. After a multiple alignment with ClustalW 1.6, gap penalty and length set at 10, the standard CLUSTREE algorithm was used for computation (Fig. 4). Interestingly, this analysis demonstrates that the mammalian 2P/4TM potassium channels reported to date that have been non-functional in standard expression systems, including murine Kcnk8, are closely related (KCNK1, KCNK6, KCN7a,b,c, and KCNK8, Fig. 4).

The genomic organization of the mammalian 2P/4TM potassium channel family was analyzed using the available databases. The first intron/exon boundary in the *Kcnk8* gene, encoding the region of the first P domain, is conserved in all mammalian members of the 2P/4TM gene family described to date (Table 3).

4. Discussion

There is no concordance between the exon structure of the *Kcnk8* gene and the predicted boundaries of the pore or transmembrane domains. However, the first intron/exon boundary lies in the middle of the first pore domain at the GYG motif. This motif is the 'signature sequence' of potassium channels and it is critical for channel function (Heginbotham et al., 1994). This is the precise location of the single intron/exon boundary in the murine *Kcnk3* gene (Lopes et al., 2000), the only mammalian 2P/4TM domain potassium channel where the gene structure has been determined by standard genomic cloning and mapping, and in all ten human 2P/4TM potassium channel genes as determined by database analyses (Table 3). Remarkably, this intron/exon boundary is located at the identical location (between the first and second nucleotides of the codon for the first glycine of GYG pore signature sequence) of an intron/exon boundary in 20 of 36 *C. elegans* 2P/4TM potassium channels (Wang et al., 1999), suggesting significant evolutionary conservation. This also suggests that numerous mammalian 2P/4TM potassium channel genes have yet to be discovered (Ketchum et al., 1995; Salkoff and Jegla, 1995; Wei et al., 1996; North, 2000).

The evolutionary origin of 2P/4TM channels is unknown (Salkoff and Jegla, 1995). It has been suggested that 2P domain potassium channels may have evolved from voltage-gated potassium channels (Derst and Karschin, 1998). Interestingly, all the potassium channels of the *Shal* subgroup examined to date, including those cloned from *Drosophila*, jellyfish, and mammals, also have an intron/exon boundary between the first and second nucleotides of the codon for the first glycine of GYG pore signature sequence (Wang et al., 1999). The functional significance of this observation is unknown. The sequences of the downstream intron are not conserved, thus it seems unlikely they contain critical regulatory sequences. However, because introns may serve as boundaries of

important functional domains, this conserved intron/exon boundary may have had a role in the diversification of potassium channels throughout evolution.

The *Kcnk8* gene localizes to murine chromosome 19, band B. Murine 19B is syntenic to human chromosome 11q11-11q13, the mapped location of *KCNK7*, the human homologue of *Kcnk8* (Salinas et al., 1999). Several human diseases have been localized to 11q13 including the Bardet-Biedl syndrome, Best's vitelliform dystrophy, and variants of insulin-dependent diabetes mellitus and cerebellar ataxia.

Mapping experiments have shown that potassium channel genes, sometimes including distantly related potassium channel subfamilies, are frequently clustered throughout the genome (Lock et al., 1994). In humans, *KCNK1* and *KCNK2* are closely mapped, at 1q41-q43, as are *KCNK4* and *KCNK7*, at 11q13 (Table 3). *KCNH1* – a voltage-gated *Eag*-related potassium channel, maps to 1q32-q41. The gene encoding the 2 P domain potassium channel *KCNK6* localizes to 19q13, a region that contains several other potassium channel genes including *KCNC2* and *KCNC3* -*Shaw* related, voltage gated potassium channels, *KCNN4* - an intermediate/small conductance calcium-activated potassium channel, and *KCNA7* – a *Shaker* related, voltage gated potassium channel. The clustering of these potassium channel genes supports the hypothesis that potassium channels arose via ancient localized gene duplication events, followed by chromosomal duplications and rearrangements followed by further gene duplication (Lock et al., 1994; Derst and Karschin, 1998).

The absence of channel function when expressed in oocytes is not unique to Kcnk8. Kcnk8 was cloned in homology to Kcnk1 (hOHO-1/TWIK-1), which we (Goldstein et al., 1998) and others (Orias et al., 1997) have found to be non-functional in the *Xenopus*oocyte expression system. There are numerous putative 2P/4TM channel subunits encoded in the genome of *C. elegans* (Ketchum et al., 1995), which also do not show function when expressed in oocytes (Wei et al., 1996). These 'non-functional' channels may require additional channel subunits, modulator substances, appropriate cellular chaperones, or may be sorted to an intracellular location rather than the cell membrane.

This work provides the framework to initiate further studies of Kcnk8, including targeted disruption of the *Kcnk8* gene by homologous recombination. Such studies may yield additional insights into the function of this protein, particularly its role in ion transport.

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